CHITOBIASE AS A REPORTER ENZYME

GOVERNMENT INTEREST IN THE INVENTION

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Related Applications

This application claims priority to U.S. Provisional Patent Application Serial Number 60/159,221, filed October 13, 1999, the disclosure of which is incorporated herein by reference in its entirety.

Background of the Invention

Field of the Invention

Reporter enzymes are enzymes whose activities are easily assayed when present inside cells. In order to study the regulation of a gene whose expression is regulated by various environmental and/or cellular factors or influences, a gene encoding a reporter enzyme may be fused to the coding region or to the regulatory region of the regulated gene.

One of the most popular cytoplasmic reporter enzymes for use in bacteria is ß-galactosidase. It is widely used in the art; however, because bacteria such as *Escherichia coli* contain an endogenous ß-galactosidase encoding gene and ß-galactosidase may be present in the cytoplasm of such bacteria, deletions of the LacZ gene, the source of the enzyme, must be introduced into the host cell line prior to its use. One goal of the present invention is to provide an alternative intracellular enzyme for use as a reporter.

This invention relates to genetic constructs and methods of using a cytoplasmic form of the chitobiase enzyme as a reporter. The invention also comprises expression vectors which express the cytoplasmic form of chitobiase. As used herein, all instances of the terminology "chitobiase" refer to a form of chitobiase which is present in the cytoplasm of the cell. Cytoplasmic forms of chitobiase may be generated via genetic engineering or microbial selection techniques.

Summary of the Invention

One embodiment of the present invention is a method for characterizing a promoter comprising providing a construct comprising the promoter operably linked to a nucleic acid encoding a cytoplasmic form of chitobiase, introducing the construct into host cells, and identifying sequences in the promoter which regulate transcription levels. In one aspect of this embodiment, the cytoplasmic form of chitobiase lacks a signal sequence. In another aspect of this embodiment, the nucleic acid encoding a cytoplasmic form of chitobiase encodes a fusion protein which comprises a cytoplasmic form of chitobiase fused to a heterologous polypeptide. In still another aspect of this embodiment, the nucleic acid encoding a cytoplasmic form encodes a cytoplasmic form of chitobiase obtained from an organism selected from the group consisting of *Alteromonas sp. 0-7, Arabidopsis thaliana, Bacillus subtilis, Bombyx mori, Bos taurus, Caenorhabditis elegans, Candida albicans, Dictyostelium discoideum, Entamoeba histolytica, Felis catus, Homo sapiens, Korat cats, Lactobacillus casei, Leishmania donovani, Mus musculus, Pisum sativum, Porphyromonas gingivalis, Pseudoalteromonas sp. S9, Rattus norvegicus, Serratia marcescens, Streptomyces plicatus, Streptomyces thermoviolaceus, Sus scrofa, Trichoderma harzianum, Vibrio furnissii, Vibrio harveyi , Vibrio parahaemolyticus,* and *Vibrio vulnificus*. In a further aspect of this embodiment, the method of identifying sequences which are involved in directing transcription comprises mutagenizing the promoter. In another aspect of this embodiment, the method of identifying sequences which are involved in transcription comprises constructing deletions in the promoter.

Another embodiment of the present invention is a method for identifying a regulatory element capable of directing or regulating transcription within a test nucleic acid sequence comprising providing a construct comprising the test nucleic acid sequence operably linked to a nucleic acid encoding a cytoplasmic form of chitobiase, introducing the construct into host cells, and determining the level of chitobiase activity. In one aspect of this embodiment, the cytoplasmic form of chitobiase lacks a signal sequence. In another aspect of this embodiment, the nucleic acid encoding a cytoplasmic form of chitobiase encodes a fusion protein, the fusion protein comprising a cytoplasmic form of chitobiase fused to a heterologous polypeptide. In still another aspect of this embodiment, the nucleic acid encoding a cytoplasmic form encodes a

cytoplasmic form of chitobiase obtained from an organism selected from the group consisting of *Alteromonas sp. 0-7, Arabidopsis thaliana, Bacillus subtilis, Bombyx mori, Bos taurus, Caenorhabditis elegans, Candida albicans, Dictyostelium discoideum, Entamoeba histolytica, Felis catus, Homo sapiens, Korat cats, Lactobacillus casei, Leishmania donovani, Mus musculus, Pisum sativum, Porphyromonas gingivalis, Pseudoalteromonas sp. S9, Rattus norvegicus, Serratia marcescens, Streptomyces plicatus, Streptomyces thermoviolaceus, Sus scrofa, Trichoderma harzianum, Vibrio furnissii, Vibrio harveyi , Vibrio parahaemolyticus,* and *Vibrio vulnificus*. In one aspect of this embodiment, the reporter gene construct is introduced transiently. In another aspect of this embodiment, the reporter gene construct is introduced stably. The host cells may be selected from the group consisting of prokaryotic cells and eukaryotic cells. In another aspect of this embodiment, the method further comprises permeabilizing or lysing the host cells. The permeabilizing or lysing step may comprise treating the host cells with toluene. The step of determining the level of chitobiase activity may be selected from the group consisting of measuring the amount of a chemiluminescent product produced from a substrate, measuring the amount of a fluorescent product produced from a substrate, measuring the amount of light absorbed by a product produced from a substrate and measuring a decrease in the amount of a detectable substrate. In another embodiment, the step of determining the level of chitiobiase activity may comprise determining the level of *p*-nitrophenol released from a substrate. In another aspect of this embodiment, the test nucleic acid sequence comprises a portion of genomic DNA. In a further aspect of this embodiment, the step of determining the level of chitobiase activity comprises determining the level of chitobiase activity after exposing the host cells to a desired set of environmental conditions. In still another aspect of this embodiment, the step of determining the level of chitobiase activity comprises determining the level of chitobiase activity after contacting the host cells with a compound to be tested for its influence on the level of transription from siad regulartory element.

Another embodiment of the present invention is a method of detecting successful transformation, comprising the steps of introducing a nucleic acid encoding a cytoplasmic form of chitobiase into host cells, and detecting chitobiase expression in the host cells.

Another embodiment of the present invnetion is a fusion protein-reporter gene construct comprising a promoter operably linked to a nucleic acid encoding a cytoplasmic form of chitobiase fused in frame with a nucleic acid encoding a heterologous polypeptide, wherein the heterologous polypeptide is not β-galactosidase or a portion thereof, and wherein the heterologous polypeptide does not contain a signal peptide. In one aspect of this embodiment, the nucleic acid encodes a cytoplasmic form of chitobiase obtained from an organism selected from the group consisting of *Alteromonas sp. 0-7, Arabidopsis thaliana, Bacillus subtilis, Bombyx mori, Bos taurus, Caenorhabditis elegans, Candida albicans, Dictyostelium discoideum, Entamoeba histolytica, Felis catus, Homo sapiens, Korat cats, Lactobacillus casei, Leishmania donovani, Mus musculus, Pisum sativum, Porphyromonas gingivalis, Pseudoalteromonas sp. S9, Rattus norvegicus, Serratia marcescens, Streptomyces plicatus, Streptomyces thermoviolaceus, Sus scrofa, Trichoderma harzianum, Vibrio furnissii, Vibrio harveyi , Vibrio parahaemolyticus,* and *Vibrio vulnificus*. In another aspect of this embodiment, the nucleic acid further comprises a λ site-specific recombination sequence.

Another embodiment of the present invention is a reporter gene construct comprising plasmid pJMF3.

Another embodiment of the present invention is a reporter gene construct comprising plasmid pJMF4.

Another embodiment of the present invention is a reporter gene construct comprising plasmid pDYK9.

Another embodiment of the present invention is a reporter gene construct comprising plasmid pDYK11.

Another embodiment of the present invention is a host cell comprising a fusion protein-reporter gene construct comprising a promoter operably linked to a nucleic acid encoding a cytoplasmic form of chitobiase fused in frame with a nucleic acid encoding a heterologous polypeptide, wherein the heterologous polypeptide is not β-galactosidase or a portion thereof, and wherein the heterologous polypeptide does not contain a signal peptide. In one aspect of this embodiment, the nucleic acid is integrated into a chromosome of the cell. In another aspect of this embodiment, the nucleic acid is transiently expressed in the host cell.

Another embodiment of the present invention is a nucleic acid encoding a cytoplasmic form of chitobiase in which the signal sequence of native chitobiase has been inactivated or deleted. In one aspect of this embodiment, the signal sequence has been mutated to inactivate it.

An isolated or purified polypeptide comprising a cytoplasmic form of chitobiase fused in frame with a heterologous polypeptide, wherein the heterologous polypeptide is not β-galactosidase or a portion thereof and wherein the heterologous polypeptide does not contain a signal peptide.

Another embodiment of the present invention is an isolated or purified polypeptide comprising a cytoplasmic form of chitobiase in which the signal peptide of native chitobiase has been inactivated or deleted. In one aspect of this embodiment, the signal sequence has been mutated to inactivate it.

Another embodiment of the present invention is a method for monitoring the activity of a promoter comprising providing a construct comprising the promoter operably linked to a nucleic acid encoding a cytoplasmic form of chitobiase, introducing the construct into host cells, and determining the level of chitobiase activity. In one aspect of this embodiment, the cytoplasmic form of chitobiase lacks a signal sequence. In another aspect of this embodiment, the nucleic acid encoding a cytoplasmic form of chitobiase encodes a fusion protein, the fusion protein comprising a cytoplasmic form of chitobiase fused to a heterologous polypeptide. In one aspect of this embodiment, the nucleic acid encoding a cytoplasmic form encodes a cytoplasmic form of chitobiase obtained from an organism selected from the group consisting of *Alteromonas sp. 0-7, Arabidopsis thaliana, Bacillus subtilis, Bombyx mori, Bos taurus, Caenorhabditis elegans, Candida albicans, Dictyostelium discoideum, Entamoeba histolytica, Felis catus, Homo sapiens, Korat cats, Lactobacillus casei, Leishmania donovani, Mus musculus, Pisum sativum, Porphyromonas gingivalis, Pseudoalteromonas sp. S9, Rattus norvegicus, Serratia marcescens, Streptomyces plicatus, Streptomyces thermoviolaceus, Sus scrofa, Trichoderma harzianum, Vibrio furnissii, Vibrio harveyi , Vibrio parahaemolyticus,* and *Vibrio vulnificus*. In another aspect of this embodiment, the reporter gene construct is introduced transiently. In still another aspect of this embodiment, the reporter gene construct is introduced stably. In a further aspect of this embodiment, the host cells are selected from the group consisting

of prokaryotic cells and eukaryotic cells. In yet another aspect of this embodiment, the method further comprises permeabilizing or lysing the host cells. For example, the permeabilizing or lysing step may comprise treating the host cells with toluene. In yet another aspect of this embodiment, the step of determining the level of chitobiase activity may be selected from the group consisting of measuring the amount of a chemiluminescent product produced from a substrate, measuring the amount of a fluorescent product produced from a substrate, measuring the amount of light absorbed by a product produced from a substrate and measuring a decrease in the amount of a detectable substrate. In still another aspect of this embodiment, the step of determining the level of chitiobiase activity comprises determining the level of *p*-nitrophenol released from a substrate. In still another aspect of this embodiment, the step of determining the level of chitobiase activity comprises determining the level of chitobiase activity after exposing the host cells to a desired set of environmental conditions. In a further aspect of this embodiment, the step of determining the level of chitobiase activity comprises determining the level of chitobiase activity after contacting the host cells with a compound to be tested for its influence on the level of transription from siad regulartory element. For example, the compound may comprise a compound to be tested for activity as a drug.

Brief Description of the Drawings

Figure 1 illustrates plasmids pJMF3 and pJMF4 containing *attP* in 2 different orientations and the *lac* promoter with the first 21 amino acids of *lacZα* (from pUC19) fused in-frame to the *chb* gene. The sequence of the fusion region is shown in Figure 2. Restriction sites shown are found once in the plasmid sequences except for *Not*I, which has 2 sites flanking the P15A origin.

Figure 2 illustrates the sequence of the *lac* promoter and the chitobiase fusion found in pJMF3 and pJMF4 (SEQ ID NOS: 15 and 16). Fusion between *lacZα* (from pUC19) and *chb* [Soto-Gill, R.W. et al, J. Biol. Chem. 264:14778-14782 (1998)] is indicated by (/); start of transcription is indicated by (+1). Sequence and binding sites in the *lac* promoter regulatory region are found in Dickson, R.C. et al., Science 187:27-32 (1975), the disclosure of which is incorporated herein by reference in its entirety. Restriction enzyme sites shown in Figure 2 are found once in the plasmid sequences except for *Sph*I, which has 2 sites; these different restriction sites can be used to replace

the *lac* promoter with another promoter together with part of a coding region to produce an in-frame fusion with *chb*.

Figure 3 depicts integration of *chb* fusions into the chromosome by site-specific recombination between *attB* and *attP*. The steps involved are described below and in Diederich, L.L, et al., Plasmid 28:14-24 (1992), the disclosure of which is incorporated herein by reference in its entirety.

Figure 4 illustrates plasmids pDYK9 and pDYK11 which contain *dnaA-chb* fusions. pDYK9 is deleted for the *rpmH* regulatory region. The orientation of *attP* in pDYK9 and pDYK11 is the same as that of pJMF3. After integration at *attB* of the larger *Not*I fragment, the *dnaA* promoters are oriented to transcribe in the same direction as replication fork movement.

Figure 5 illustrates the *rmpH-dnaA* regulatory region and *dnaA*-chitobiase fusion (SEQ ID NO: 17). The *dnaA* box and promoters are shaded, and the coding region of the *dnaA* gene is striped. The fusion contains two amino acids (between the backslashes) from pUC19. The region cloned into pDYK9 is between *Sph*I primer II and *Kpn*I primer, and the region cloned into pDYK11 is between *Sph*I primer I and *Kpn*I primer. The numbers above the primers refer to the nucleotide in the sequence amplified. For sequence numbering and the locations of promoters and protein binding sites, see Froelich, J.M. et al., J. Bacteriol. 178:6006-6012 (1996); Hansen, F.G. et al., EMBO J. 1:1043-1048 (1982) and Messer, W. and C.W. Weigel, Initiation of Chromosome Replication. p. 1579-1601 (1996) In F.C. Neidhart, R. Curtiss III, J.L. Ingraham, E.CC. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter and H.E. Umbarger (Eds.), Escherichia coli and Salmonella Cellular and Molecular Biology., ASM Press, Washington, D.C., the disclosures of which are incorporated herein by reference in their entireties.

Detailed Description of the Preferred Embodiment

Reporter genes and reporter gene constructs play a number of important roles in a variety of molecular biology techniques. For example, reporter genes may be used to determine whether a sequence contains a promoter or other cis-acting element which directs transcription, such as an enhancer. In addition, reporter genes may be used to identify regulatory sites in promoters or other cis-acting elements and to determine the

effects of mutating these regulatory sites on the level of gene expression directed by the promoters or other cis-acting elements. Reporter genes may also be used to detect successful transformation. In addition, reporter genes may be used to monitor gene expression under various conditions and to identify drugs.

The structure of a reporter gene construct containing the cytoplasmic form of chitobiase will vary according to its purposes. When the reporter gene construct is a vector, one must decide between a vector that incorporates the reporter gene into the host's genome or one that replicates extrachromosomally, such as a plasmid. When the reporter construct is not designed to integrate into the host genome, the vector can contain an origin of replication with activity in the host cell of interest. This feature provides the reporter gene vector the ability to replicate within the host cell in which it has been introduced. In addition to the origin of replication, reporter constructs often contain a promoter, a multiple cloning site, a selectable marker, and of course a reporter gene. Reporter constructs for use in eukaryotic cells may also contain a polyA site adjacent to the reporter gene.

Given the utility of reporter gene constructs, it is not surprising that a number of cytoplasmic reporter gene constructs and different reporter genes are available for use by those of skill in the art. For example, the cytoplasmic reporter enzymes chloramphenicol acetyltransferase (CAT), firefly luciferase, β-glucuronidase (GUS), green fluorescent protein (GFP), and ß-galactosidase have been used extensively. However, such reporters all have individual shortcomings that may limit or preclude their usage under some conditions. For example, high levels of GFP are toxic to the cell. In addition, reporter enzymes are not expressed equally in all cell types nor are they equally stable when expressed in all cell types. Furthermore, there is a recognized need for multiple reporter enzymes that can be assayed independently of one another in order to simultaneously study the regulation of multiple genes within a single cell type. Therefore, there exists a continuing need to identify reporter enzymes with useful properties.

The cytoplasmic enzyme ß-galactosidase is widely used as a reporter gene in various microbiological and molecular biological studies. This enzyme is used in both *in vitro* and *in vivo* assays. The wide acceptance of this reporter system results, in part, because it is non-isotopic and extremely flexible. It is used in a number of assay

formats and has an extremely broad linear range. Nevertheless, because ß-galactosidase is present in the cytoplasm of various host cells such as *Escherichia coli*, deletion of the *lacZ* gene, the source of the enzyme, is often required prior to its use in a host cell system. One goal of the present invention was to provide an alternative intracellular enzyme for use as a reporter.

An extensive discussion of various molecular biology techniques is available in Ausubel, et al., (eds) "Short Protocols in Molecular Biology," Wiley and Sons, Inc., New York (1997), the disclosures of which are incorporated herein by reference in their entireties. Examples of such techniques include isolating and preparing DNA for manipulation, gel electrophoresis, polymerase chain reaction (PCR), determining nucleic acid sequences, screening nucleic acid libraries, mutagenesis of DNA, and introducing DNA into host cells.

The structure of a reporter gene construct will vary according to its purposes. The reporter gene constructs are constructed according to standard techniques of molecular biology well known in the art.

When the reporter gene construct is a vector, one must decide between a vector that incorporates the reporter gene into the host's genome or one that replicates extrachromosomally, such as a plasmid. When integration of the reporter gene is a desired result, a reporter gene construct will contain sequences that will facilitate incorporation.

One example of integration sequences that can be included in a reporter gene construct is the λ *attP* site. This site permits a single copy of the reporter gene to be incorporated into a host bacterial genome. Integration-promoting sequences with utility in mammalian cells include the long terminal repeats found in retroviral genomes. These sequences promote viral genome integration in a host genome and have been used extensively by those of skill in the art to promote the integration of exogenous sequences in mammalian host cells.

When the reporter construct is not designed to integrate into the host genome, it is common that the vector contain an origin of replication with activity in the host cell of interest. This feature provides the reporter gene vector the ability to replicate within the host cell in which it has been introduced.

In addition to the origin of replication, reporter constructs will often contain additional features that promote the expression of the nucleic acid sequence or sequences contained in the construct. These additional sequences can include a polyA site, a multiple cloning site, a drug resistance marker, and of course a reporter gene.

The present invention relates to the use of chitobiase as a reporter gene. The chitobiase may be used as a reporter in bacteria, plants, mammalian cells and other host cell lines. One possible alternative to using ß-galactosidase as a reporter gene was to develop cytoplasmic N,N'-diacetylchitobiase (*N*-acetyl-ß-D-glucosaminidase, EC 3.2.1.30) for use as a reporter enzyme. One advantage of the enzyme *N*-acetyl-ß-D-glucosaminidase or "chitobiase" over ß-galactosidase is that genes encoding chitobiase are missing from many bacteria, including *E. coli*, some fungi, and some eukaryotic cells. Thus, it is not necessary to engineer many host cells to lack reporter activity as is the case with ß-galactosidase. The present invention also relates to various protein expression vectors that can be used to express the reporter gene. In addition, the present invention may be used in conjunction with other reporter enzymes in systems in which the regulation or activities of multiple genes is to be studied simultaneously.

Chitobiase is one of two enzymes that hydrolyze chitin, an abundant insoluble polysaccharide, to its monomeric unit, *N*-acetylglucosamine (GlcNac). Chitobiase is known to be present in a number of organisms. For example, the chitobiase enzyme is known to be present in various genera including *Arabidopsis, Bacillus*, *Bombyx, Bos, Caenorhabditis*, *Candida, Dictyostelium, Entamoeba, Felis, Homo, Korat, Lactobacillus, Leishmania, Mus*, *Pisum, Porphyromonas, Pseudoalteromonas, Rattus, Serratia, Streptomyces, Sus*, *Trichoderma,* and *Vibrio*. Specific examples of organisms known to contain chitobiase include *Alteromonas sp. 0-7, Arabidopsis thaliana, Bacillus subtilis, Bombyx mori, Bos taurus, Caenorhabditis elegans, Candida albicans, Dictyostelium discoideum, Entamoeba histolytica, Felis catus, Homo sapiens, Korat cats, Lactobacillus casei, Leishmania donovani, Mus musculus, Pisum sativum, Porphyromonas gingivalis, Pseudoalteromonas sp. S9, Rattus norvegicus, Serratia marcescens, Streptomyces plicatus, Streptomyces thermoviolaceus, Sus scrofa, Trichoderma harzianum, Vibrio furnissii, Vibrio harveyi , Vibrio parahaemolyticus,* and *Vibrio vulnificus*.

One source of the enzyme is the marine bacterium, *Vibrio harveyi*. *Escherichia coli* cells harboring a plasmid carrying the *chb* gene from *Vibrio harveyi* were reported to produce the enzyme, which was found to be associated with the outer membrane of the bacterial cells. These are described in Jannatipour, M. et al., "Translocation of *Vibrio harveyi N,N'*-diacetylchitobiase to the outer membrane of *Escherichia coli*.," J. Bacteriol. *169*:3785-3791 (1987) and Soto-Gil & Zyskind, *N,N'*-Diacetylchitobiase of *Vibrio harveyi* primary structure, processing, and evolutionary relationships. J. Biol Chem. *264*:14778-14782 (1989), both of which are hereby incorporated by reference.

The present invention contemplates expressed cytoplasmic forms of chitobiase in various forms. In one embodiment, the signal sequence is deleted from the amino terminal portion of the protein. Presumably the removal of this sequence results in the expression of a cytoplasmic form of the enzyme that is not secreted from the host cell or incorporated into the membrane of the host cell producing the enzyme.

The present invention also contemplates the generation of fusion proteins comprising a fusion polypeptide joined in frame to chitobiase. Preferably, the fusion polypeptide comprises a polypeptide other than chitobiase, such as a heterologous protein. The heterologous polypeptide may comprise a polypeptide having a biological activity (such as an enzymatic or other activity besides activity as an immunogen) or the heterologous polypeptide may not have a biological activity. The heterologous polypeptide does not include a signal sequence which directs its secretion. Preferably the heterologous polypeptide is not β-galactosidase or a portion thereof. Thus, the fusion reporter gene construct contains a sequence encoding the fusion polypeptide genetically fused in frame with a sequence encoding chitobiase. In one embodiment, this fusion may remove the amino-terminal signal peptide sequence of chitobiase and replace it with a heterologous protein.

In another embodiment, the fusion protein construct comprises a chitobiase gene sequence that has been truncated to remove at least the signal peptide sequence of the gene. Alternatively, mutations may be introduced into the signal peptide sequence so that it is no longer functional. Such mutations may be introduced using a variety of techniques familiar to those skilled in the art, including site directed mutagenesis, cassette mutagenesis, and chemical mutagenesis.

Once the reporter gene construct is made it is introduced into a host cell line for testing. Host cells of prokaryotic and eukaryotic origin can be used with the reporter gene constructs of the present invention. A variety of methods are available to introduce reporter gene constructs into host prokaryotic cells. For example, bacteria can be transformed using calcium chloride, electroporation, or viral vectors such as the filamentous phages. These and other prokaryotic transformation protocols are well known in the art.

Alternatively, the sequence encoding chitobiase may be introduced in eukaryotic cells, including yeast, mammalian, plant, and insect cells. For example, the sequence encoding chitobiase may be inserted into a yeast artificial chromosome, a yeast plasmid, a bovine papilloma virus vector or other extrachromosomal vector, a retroviral vector, a Ti-plasmid, or a baculovirus vector. A variety of such vectors are known to those skilled in the art. The vectors may be introduced into any of the yeast, mammalian, plant, and insect cells familiar to those skilled in the art.

The introduction of the reporter gene construct into mammalian cells can likewise utilize a number of transfection protocols well known to those of skill in the art. As discussed above, transfections can be transient or stable. Examples of suitable transfer protocols include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection, and viral transfection. These and other eukaryotic transformation protocols are well known in the art.

Following introduction of the reporter gene construct into the host cell of interest, the enzymatic activity of the reporter gene is measured. Preferably, the chitobiase assays are performed after permeabilizing or lysing the host cells. There are a variety of cell permeabilization and cell lysis procedures available to those of ordinary skill in the art, including methods such as sonication or lysozyme treatment. One embodiment of the present invention uses toluene treatment to permeabilize cells. The details of this method are discussed in D.Y. Kalabat et al., BioTechniques 25:1030-1035 (1998) and Miller , J.H. A Short Course in Bacterial Genetics, CSH Laboratory Press, Cold Spring Harbor, NY 1992, the disclosures of which are incorporated herein by reference in their entireties.

Cellular chitobiase activity can be measured quantitatively by following the hydrolysis of chitobiase substrates. Examples of substrates with utility in chitobiase

activity assays include *N,N'*-diacetylchitobiose (chitobiose), *p*-nitrophenyl-*N*-acetyl-ß-D-glucosaminide (PNAG)(Sigma Chemical, St. Louis, MO), and 5-bromo-4-chloro-3-indolyl-*N*-acetyl-ß-D-glucosaminide (X-Gluc)(Sigma Chemical, St. Louis, MO). Other substrates are also contemplated for use in the assays of the present invention.

Products produced by the hydrolysis of the chitobiase substrates are monitored using various means familiar to those skilled in the art. For example, various optical means are known to those skilled in the art. One such optical means may comprise detection of chemiluminescent or fluorescent products released from a substrate. Alternatively, the level of chitobiase activity may be determined by measuring the amount of light absorbed by a product produced from a substrate or measuring a decrease in the amount of a detectable substrate. In one embodiment, *p*-nitrophenol is released from the substrate and measured at 400 nm. Other monitoring methods well known in the art can be used to quantitate signals produced in the chitobiase assay. These may inlcude use of radioactive substrates or substrates having radiofrequency tags. In another embodiment, blue/white colony indicator plates are used to monitor enzyme activity.

Another embodiment of the present invention is a kit. One aspect of this embodiment includes a reporter gene construct comprising a vector containing a chitobiase reporter gene. The reporter gene construct also contains a multiple cloning site containing a variety of restriction endonuclease cutting sites that facilitate the introduction of exogenous DNA into the construct.

The kit embodiment of the present invention also includes those components necessary to assay for chitobiase activity produced by the reporter gene construct. For example, in one embodiment, the kit will include a supply of a suitable chitobiase substrate whose metabolism into product by the reporter enzyme can be assayed.

EXAMPLES

The following Examples are disclosed to assist in the understanding of the present invention. The Examples below should not be construed to limit the scope of the invention and such variations of the invention now known or later developed, which would be within the purview of one of ordinary skill in the art and are considered to fall within the scope of the invention hereinafter claimed.

Example 1

Construction of Vectors

Vectors were constructed using polymerase chain reaction (PCR) products that were cloned first at the *Sma*I or *Eco*RV restriction sites of plasmid pBluescript II (pKSII+)(Stratagene; San Diego, CA). The nucleotide sequence of all PCR products was determined using standard techniques well known in the art. Unmethylated plasmid DNA was isolated from an *E. coli dam*$^-$ strain when cutting with the *Bcl*I enzyme was required.

The pDYK9 (SEQ ID NO: 11) plasmid was constructed by ligating a *Sph*I-*Kpn*I PCR product containing the *dnaA* promoter region into plasmid pRSG196 that contains the *V. harveyi* chitobiase gene (see Jannatipour, M., et al.). Briefly, the *V. harveyi* chitobiase gene was prepared by cloning a *Sau*3A partial digest of *V. harveyi* DNA into the single *Bam*HI site with the *tet* gene of pMK2004 [See Soto-Gill & Zyskind, "Cloning of *Vibrio harveyi* chitinase and chitobiase genes in *Escherichia coli*," pp. 209-223, *In* J.P. Zikakis (ed.), Chitin, chitosan, and related enzymes," Academic Press, Inc., New York (1984)]. The clones containing the gene of interest were detected by the presence of the yellow *p*-nitrophenylate product after individual colonies of the clone bank were sprayed with 10 mM PNAG dissolved in 100 mM sodium phosphate, pH 7.0. Restriction maps of the positive clones were made according to standard techniques well known in the art.

The *chb* gene was subcloned in a 3.5-kb *Eco*RI fragment from pRSG14 [See Jannatipour, M. et al., "Translocation of *Vibrio harveyi N,N'*-diacetylchitobiase to the outer membrane of *Escherichia coli.*," J. Bacteriol. *169*:3785-3791 (1987)] into the *Eco*RI site of pUC19 [Yanisch-Perron, c.J. et al., Gene 33: 103-119 (1985)]. Plasmid pRSG196 was constructed by deleting a 0.5-kb *Sst*I fragment from one of these clones [See Jannatipour, M. et al., "Translocation of *Vibrio harveyi N,N'*-diacetylchitobiase to the outer membrane of *Escherichia coli.*," J. Bacteriol. *169*:3785-3791 (1987)].

The pRSG196 plasmid was also cut with *Sph*I and *Kpn*I to accommodate the insertion of the *dnaA* fragment. Plasmid pAC17 [described in Chiaramello & Zyskind, "Coupling of DNA replication to growth rate in *Escherichia coli*: a possible role for guanosine tetraphosphate," J. Bacteriol. 172:2013-2019 (1992) hereby incorporated by reference] served as template with primers 5'-GCA CAT GCA TGC TGG TCA TTA

AAT TTT CC-3' (SEQ ID NO 1) and 5'-CGG GGT ACC AAC TCA TCC TGC AAT CG-3' (SEQ ID NO 2) producing a PCR product 374 bp long that contains 353 bp from the *dnaA* promoter region [bases 583 to 935, numbering according to Hansen, F. G., et al., "The nucleotide sequence of the *dnaA* gene promoter and of the adjacent *rpmH* gene, coding for the ribosomal protein L34, of *Escherichia coli*," EMBO J. 1:1043-1048 (1982)]. The forward primer (*Sph*I primer II) contained an *Sph*I site and the reverse primer (*Kpn*I primer) contained a *Kpn*I site for cloning. This created an in-frame fusion between the amino-terminal 17 amino acids of DnaA and the carboxy-terminal end of chitobiase deleted for the amino-terminal 22 amino acids including the signal peptide.

The next step in constructing the pDYK9 vector was taking the 3270 bp *Dra*I-*Hin*dIII (partial digest) fragment containing the *dnaA-chb* fusion and ligating it to the pACYC184 2555 bp *Hin*cII-*Hin*dIII fragment carrying chloramphenicol-resistance (Cm^r) and the P15A origin. This fragment was described in Chang & Cohen, "Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid," J. Bacteriol. 134:1141-1156 (1987).

A *Not*I site was introduced at the *Acc*I site after digestion with *Acc*I, treatment with Mung bean nuclease, and ligation to phosphorylated *Not*I linkers (New England Biolabs, Inc.; Beverly, MA). A *Not*I site was introduced at the *Ase*I site after digestion with *Ase*I, treatment with Mung bean nuclease, and ligation to phosphorylated *Not*I linkers (New England Biolabs, Inc.; Beverly, MA). An *Xba*I-*Sph*I PCR product containing the *rrnBt1t2* terminator was ligated into this plasmid cut with the same enzymes creating pDYK7. *E. coli* chromosomal DNA served as template with primers 5'-CTA GTC TAG ATG CCG AAC TCA GAA GTG A-3' (SEQ ID NO 3) and 5'-GCA CAT GCA TGC GGG GGA TGG CTT GTA GAT-3' (SEQ ID NO 4) to produce a PCR product 357 bp long that contains bases 6534 to 6869 from the *rrnB* operon [numbering according to Brosius, J., et al., "Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli.*" J. Mol. Biol. 148:107-127 (1981)] and includes the complex transcription termination region of this operon which is described in Orosz, A., et al., "Analysis of the complex transcription termination region of the *Escherichia coli rrnB* gene," Eur. J. Biochem. *201*:653-659 (1991). The forward primer contained an *Xba*I site and the reverse primer contained an *Sph*I site.

A *Bcl*I-*Sma*I PCR product containing the λ *attP* site was ligated into pDYK7 cut with *Tth111*I, treated with Mung bean nuclease, then digested with *Bcl*I. Plasmid pHN894 [described in Goodman, S. D., et al., "Deformation of DNA during site-specific recombination of bacteriophage lambda: Replacement of IHF protein by HU protein or sequence-directed bends," Proc. Natl. Acad. USA *89*:11910-11914 (1992)] served as template with the forward primer 5'-CAT GAT CAT GCG ACA GGT TTG ATG A-3' (SEQ ID NO 5) and the reverse primer 5'-GGG GGC GCC TAC CTT TCA CGA G-3' (SEQ ID NO 6) producing a PCR product 466 bp long that contains the λ *attP* site. The 466 bp PCR product containing the λ *attP* site was first cloned into the *Sma*I site of the pKSII+ plasmid to produce pDYK8. The forward primer contains a *Bcl*I site and the reverse primer contains G's at the 5' end in order to recreate a *Sma*I site when cloning the PCR fragment into a *Sma*I site. This PCR product includes bases -211 to +241 from the center of the *attP* core and the sequence required for optimum λ *attP* site integration as described in Nagaraja & Weisberg, "Specificity determinants in the attachment sites of bacteriophages HK022 and λ," J. Bacteriol. 172:6540-6550 (1990). The orientation of *attP* is such that when the fusion is integrated at *attB*, the transcription direction of *dnaAp1* and *p2* promoters is the same as replication fork movement mimicking the orientation at the wild type *dnaA* promoters.

Vector pDYK11 (SEQ ID NO: 12) was constructed by ligating an *Sph*I-*Kpn*I PCR product containing the *rpmH-dnaA* promoter region into pDYK9 also digested with *Sph*I and *Kpn*I. Plasmid pAC17, described in Chiaramello & Zyskind, (1992), served as template with the primers 5'-CAT GCA TGC ATG AAA CGA TGG ACA CC-3' (SEQ ID NO 7) and 5'-CGG GGT ACC AAC TCA TCC TGC AAT CG-3' (SEQ ID NO 8) to produce a PCR product 616 bp long that contains 598 bp from the *rpmH-dnaA* regulatory region [bases 338 to 935, numbering according to Hansen, F. G., et al., (1982)]. The forward primer (*Sph*I primer I) contained an *Sph*I site and the reverse primer (*Kpn*I primer) contained a *Kpn*I site for cloning. SEQ ID NO: 18 provides the complete coding sequence of the DNA encoding the dnaA/chitobiase fusion protein. The complete sequence of the dnaA/chitobiase fusion protein is provided in SEQ ID NO: 19.

The vector pJMF3 (SEQ ID NO: 13) was constructed by first ligating an *Ase*I linker (5'-CATTAATGCATG-3' (SEQ ID NO 9) self-hybridized) into the *Sph*I site of

pDYK11. The pUC19, [described in Yanisch-Perron C., et al., "Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors," Gene *33*:103-19 (1985)] *Ase*I-*Kpn*I fragment containing the *lacPO*-polylinker region was ligated to this plasmid after digestion with *Ase*I and *Kpn*I. The resulting in-frame fusion between the amino-terminal 21 amino acids of the pUC19 *lacZ*(α) peptide [Yanisch-Perron C., et al., 1985] and the carboxy-terminal end of chitobiase deleted for the amino-terminal 22 amino acids is identical to the protein fusion in pRSG196 [Jannatipour, M., et al., (1995)].

Vector pJMF4 (SEQ ID NO: 14) was constructed by ligating the *Bam*HI-*Eco*RV fragment of pDYK8 containing the λ *attP* site into pDYK7 which had been digested with *Tth111*I, treated with Mung bean nuclease, then digested with *Bcl*I, to create pTKP9. This reversed the orientation of the *attP* site relative to the *attP* site in pDYK9. The pDYK11 *Bsp*MII-*Kpn*I fragment containing the *rpmH-dnaA* promoter region was then ligated into pTKP9 digested with the same enzymes to create pTKP11. This reversed the orientation of the *attP* site relative to the *attP* site in pDYK11. An *Ase*I linker (5'-CATTAATGCATG-3' (SEQ ID NO 10) self-hybridized) was ligated into the *Sph*I site of pTKP11 to create pJMF2. The pUC19 [as described in Yanisch-Perron C., et al., (1985)] *Ase*I-*Kpn*I fragment containing the *lacPO*-polylinker region was ligated to pJMF2 cut with *Ase*I and *Kpn*I to create pJMF4.

Example 2

Site-specific Recombination

To move the chitobiase fusions in pDYK9 and pDYK11 to the *attB* site in the chromosome, *Not*I fragments from these plasmids were self-ligated and transformed or electroporated into strain WM2269 (DH5α containing pLDR8) [See Zyskind, J. W. and S. I. Bernstein (1992) "Recombinant DNA Laboratory Manual," Academic Press, San Diego, CA]. Plasmid pLDR8, described in Diederich, L. L. J., et al., "New cloning vectors for integration into the λ attachment site *attB* of *Escherichia coli* chromosome," Plasmid *28*:14-24 (1992), expresses integrase from the λ P_R promoter and contains the λ cI_{857} repressor gene, a kanamycin resistance gene, and a temperature-sensitive origin of replication. The transformed or electroporated cells were incubated at 42°C with shaking for 30 min then moved to 37°C for 1 h followed by selection on Luria broth

agar plates containing chloramphenicol (25 μg/ml) at 42°C. Transformants were screened for loss of kanamycin resistance and, therefore, loss of pLDR8.

Example 3

Bacteriophage P1 Transduction

Transduction with P1 bacteriophage by the method of Zyskind & Bernstein, Recombinant DNA Laboratory Manual. Academic Press, San Diego, CA (1992) was used to construct strains and to confirm the chromosomal location of the *dnaA-chb* fusions. Cotransduction of Cm^r (carried by the fusion) and *galK* (linked to *attB*) indicated that Cm^r and *galK* are linked on the chromosomes of strains DYK9W, DYK9F, DYK11W, and DYK11F.

Example 4

p-Nitrophenol Chitobiase Activity Assay

Chitobiase activity is located in the cytoplasm when its signal peptide is replaced by fusion with another peptide. Accordingly, chitobiase assays are performed on permeabilized or lysed cells. A variety of permabilization or cell lysis protocols are available to liberate the enzyme from within a cell population being tested. One such protocol involves toluene-treated cells washed once with M9 salts according to the method of Miller, J.H. A Short Course in Bacterial Genetics, CSH Laboratory Press, Cold Spring Harbor, NY 1992, which is hereby incorporated by reference. The toluenized cells are placed in a chitobiase buffer (10 mM Tris-HCl, pH 8.0, and 0.5 M NaCl) and 666 μM PNAG, the chitobiase enzyme substrate. NaCl is included because chitobiase has approximately 80% of full activity in the absence of salt, with maximal activity occurring between 0.25 and 0.6 M NaCl. Toluenized cells (0.772 ml) are preincubated at 28°C and the reaction started with the addition of 0.228 ml PNAG (1 mg/ml). After incubation at 28°C, the reaction is stopped by the addition of 1 ml of 1 M Tris base. The release of *p*-nitrophenol is measured at 400 nm and turbidity at 550 nm. *p*-Nitrophenol release is measured immediately at 400 nm with a molar absorptivity of 1.8×10^3 liters mol^{-1} cm^{-1}. Units are calculated after subtracting the light scattering factor ($1.5 \times OD_{550}$) from OD_{400} of the sample. The normalizing factor of 1.5 was determined previously by measuring the light scattering ratio of bacteria at OD_{400} and OD_{550}. One unit of chitobiase activity is the amount of enzyme that catalyzes the formation of 1 pmol of *p*-nitrophenol per min at 28°C. For comparison to Miller units

of ß-galactosidase [described in Miller, J. H., A Short Course in Bacterial Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1992)], the units are normalized to 1 ml of culture at OD_{450}=1.

Example 5

Determination of Chitobiase Activity in Cells Containing Vectors pJM3 and pJM4

(Figure 1) contain the *lacPO* promoter with the first 21 amino acids of *lacZα* (from pUC19) fused in-frame to the *chb* gene. These plasmids also contain the λ phage *attP* recombination site in different orientations, the gene encoding chloramphenicol acetyltransferase (*cat*), and a ribosomal terminator, *rrnBt1t2*, inserted upstream of the *lac-chb* fusion to prevent read-through from other promoters. The chitobiase activity associated with these plasmids (Table 1) is high in the absence of IPTG because of titration of *lac* repressor expressed from a single copy chromosomal gene. Induction by IPTG is approximately 10-fold (Table 1).

Table 1. Chitobiase Activity of *lacZ-chb* Fusion[a]

	Chitobiase[b] (U[c])	
Plasmid	**-IPTG**	**+IPTG (1 mM)**
pJMF3 in DH5α	668 ± 45	9320 ± 347
pJMF4 in DH5α	788 ± 44	7188 ± 477

[a] Overnight cultures were diluted 1:1000 into 50 mL prewarmed LB and grown to OD_{450} = 0.1. 1 mM IPTG was added to half of the culture, and growth continued to OD_{450} = 0.3.
[b] Triplicate samples were assayed. Mean chitobiase activities are given with standard deviations.
[c] One unit of chitobiase activity is 1 pmol of *p*-nitrophenol/min at 28°C. Units given for 1 mL of culture at OD_{450} = 1.

The *lac* promoter can be replaced with another promoter and a fusion protein created with chitobiase by cutting with *Sph*I or *Ase*I and either *Sal*I, *Kpn*I, or *Sst*I (Figure 2). Fusions created with these vectors can be moved to the chromosome by site specific recombination at the λ *attB* site to permit single copy analysis of the activity of the promoter. The protocol, as described more fully in Diederich, et al., "New cloning vectors for integration into the λ attachment site *attB* of *Escherichia coli* chromosome,"

Plasmid *28*:14-24 (1992), involves two components, (i) a circular DNA containing the λ attachment site, *attP*, the promoter-*chb* gene fusion, and the *cat* gene, and (ii) a helper plasmid, pLDR8, which contains the *int* gene under the control of the temperature-sensitive repressor, *cI857* and a temperature-sensitive origin of replication. The plasmid is digested with *Not*I to remove the P15A origin, and the fragment containing the chitobiase fusion is self-ligated prior to transformation into cells containing pLDR8. Integration occurs by site-specific recombination between *attP* and *attB* (17.4 min on the *E. coli* chromosome, Figure 3).

Example 6

Use of the Chitobiase Reporter Enzyme to Study dnaA Gene regulation

Two plasmids, pDYK9 and pDYK11, discussed in the Examples above, were constructed to assess the regulation of the *dnaA* gene using chitobiase as a reporter enzyme. These plasmids differ by the absence of the *rpmH* regulatory region in pDYK9 (Figure 4). These fusions were moved from the plasmid to the chromosomal *attB* site for single copy analysis as described above. After transformation of strain WM2269 with the ligated DNA, integration occurred by site specific recombination between the *attP* and the *attB* sites. The orientation of *attP* in pDYK9 and pDYK11 is such that when the fusion is integrated at *attB*, the transcription direction of *dnaAp1* and *dnaAp2* promoters is the same as movement of the replication fork. This orientation is the same as at the *dnaA* wild type location. The genetic location of the fusions was confirmed in the Cm^r transformants by demonstrating cotransduction of Cm^r and *galK*.

The fusions created in strain WM2269 were moved by P1 transduction to MG1655, creating strain DYK9W with pDYK9 and strain DYK11W with pDYK11. Deletion of the *rpmH* promoters had very little effect (1.4-fold) on chitobiase activity (Table 2, compare lines 1 and 3)

Table 2. Chitobiase and β-Gal Activities of *dnaA-chb* and *dnaA-lacZ* Fusions in WT and *fis* Mutant Backgrounds

Strains	Chitobiase[a] (U[b])	β-Gal[c] (Miller U[d])
DYK11W *fis*+	30.0 ± 2.1	
DYK11F *fis*::985	80.0 ± 1.2	
DYK9W *fis*+	44.0 ± 2.1	
DYK9F *fis*::985	96.0 ± 3.5	
RB220 *fis*+		59.8 ± 7.9
TP220 *fis*::767		115.2 ± 7.2

[a] Triplicate samples were assayed during exponential growth. Mean chitobiase activities are given with standard deviation.
[b] One unit of chitobiase activity is 1 pmol of *p*-nitrophenol per min at 28°C. Units given for 1 mL of culture at $OD_{450} = 1$.
[c] Data from Reference 8.
[d] Unit defined in Reference 13.

Fis protein binds to a site in the *dnaAp2* promoter that covers the -35 sequence (Figure 5), and appears to be a repressor of DnaA expression. A fusion protein with ß-galactosidase activity that is expressed from the *rpmH-dnaA* regulatory region has increased ß-galactosidase activity (1.9-fold) in a *fis*⁻ mutant when compared to Fis wild type cells [as described in Froelich, J. M., et al., "Fis binding in the *dnaA* operon promoter region," J. Bacteriol. *178*:6006-6012 (1996), data shown in Table 3]. Similarly, the absence of Fis leads to a greater than 2-fold increase in chitobiase activity of the DnaA-chitobiase fusion protein for the DYK9F and DYK11F strains, comparable in extent to that observed with the *dnaA-lacZ* fusion strain, TP220 (Table 3).

In the reporter gene constructs discussed in the Example, all upstream transcriptional activity was prevented from entering the *chb* reporter gene. The plasmid vectors, pJMF3 and pJMF4, described in the Examples above, contained the *rrnbt1t2* terminator upstream of the promoter fusion, which prevented readthrough from chromosomal promoters near the insertion site. Only chitobiase activity originating from the promoters of interest was expressed.

The *attP* site in these vectors allowed integration at the chromosomal *attB* in a specific orientation, depending on the vector used. With these vectors, any chitobiase fusion involving an essential gene can be moved to the chromosome, thus permitting single copy analysis with a chromosomal orientation similar to the wild-type gene.

Example 7

Identification of Promoters in Test Sequences

A nucleic acid prospectively containing a promoter is inserted upstream of a nucleic acid encoding a cytoplasmic form of chitobiase as described above. For example, the nucleic acid prospectively containing a promoter may be inserted into a restriction site in a sequence containing a plurality of restriction sites, such as a polylinker, which is located upstream of the nucleic acid encoding chitobiase. The test sequence may comprise any nucleic acid to be tested for promoter activity. In one embodiment, the test sequence may comprise a genomic DNA sequence. For example, the genomic DNA sequence may be a randomly generated DNA fragment, such as a fragment generated using shotgun cloning techniques, a restriction fragment, or any other sequence.

The vectors containing the test sequence upstream of the nucleic acid encoding chitobiase are introduced into an appropriate host cell. The level of chitobiase activity is assayed and compared to the level obtained from a control vector which lacks an insert in the cloning site. The presence of an elevated expression level in cell containing the vector containing the insert with respect to the level in cells containing the control vector without the insert indicates the presence of a promoter in the insert.

In some embodiments, the activity of the promoter in the test sequence may be assayed after exposure of the host cells to conditions which may influence the level of transcription from the promoter. For example, the environment of the host cells may be altered to determine whether the transcription level is influenced by environmental factors, including factors such as temperature, pH, nutrients, or availability of oxygen. In such analyses, chitobiase levels are assayed under a variety of environmental conditions to determine the effects of the environmental conditions on transcription levels from the promoter. In addition, the activity of the promoters may be examined in the presence or absence of compounds to be tested for regulatory activity. For example, the activity of the promoters may be tested by determining the levels of chitobiase produced in the presence or absence of compounds to be tested for activity as drugs.

Promoter sequences within the test sequences may be further defined by constructing nested deletions in the test sequences using conventional techniques such as Exonuclease III digestion. The resulting deletion fragments can be inserted into the promoter reporter vector to determine whether the deletion has reduced or obliterated promoter activity as determined by measuring chitobiase activity in cells containing the deletion vectors. In this way, the boundaries of the promoters may be defined. If desired, potential individual regulatory sites within the promoter may be identified using techniques such as site directed mutagenesis or linker scanning to obliterate potential transcription factor binding sites within the promoter individually or in combination. The effects of these mutations on transcription levels may be determined by inserting the mutations into the cloning sites in the promoter reporter vectors and measuring the levels of chitobiase produced from the mutated promoters.

The activity of known promoters may also be monitored by operably linking them to a nucleic acid encoding a cytoplasmic form of chitobiase. The activity of the promoters may be analyzed under various environmental conditions as described above. In addition, the activity of the promoters may be analyzed in the presence or absence of compounds to be tested for the ability to affect transcription from the promoters. For example, the compounds may be tested for activity as drugs.

In some embodiments, the chitobiase reporter constructs may be used in systems for identifying compounds that modulate cell surface protein-mediated activity or compounds which modulate the activities of intracellular signaling systems. Techniques for using reporter genes to identify compounds which modulate cell surface protein-mediated activity have been described in U.S. Patent Number 5,401,629 and U.S. Patent Number 5,436,128, the disclosures of which are incorporated herein by reference in their entireties. Briefly, in such methods, a construct comprising a promoter operably linked to a nucleic acid encoding a reporter enzyme is introduced into cells which express the cell surface protein and cells which do not express the cell surface protein. Each of the cells are contacted with test compounds and the effects of these compounds on transcription levels is measured by determining the level of activity of the reporter enzyme. The level of expression of the reporter gene in cells expressing the cell surface protein is compared to the level in cells which do not express the cell surface protein to identify compounds that modulate cell surface protein activity.

Similarly, the chitobiase reporter constructs may be used to identify compounds which influence the activity of intracellular signaling pathways, such as cAMP-based or phosphorylation-based pathways. In such methods, a promoter which is activated via such pathways is operably linked to a nucleic acid encoding a cytoplasmic form of chitobiase. The cells are contacted with test compounds. Those compounds which activate the pathway to which the promoter responds will produce an enhanced level of chitobiase activity in the cells as compared to the level of chitobiase activity in control cells which have not been contacted with the test compound.

Example 8

Detecting Successful Transformation or Transfection Using Chitobiase

A vector comprising a sequence encoding a cytoplasmic form of chitobiase operably linked to a sequence capable of directing transcription of the chitobiase gene is introduced into a host cell. The host cells are contacted with a chitobiase substrate and those host cells which contain chitobiase activity are identified as cells which were successfully transformed or transfected. In some embodiments, a portion or replica of a colony may be lysed or permeabilized prior and the lysate or permeabilized cells may be contacted with the chitobiase substrate.

CONCLUSION

New gene reporter systems that use chitobiase have been described. Chitobiase has advantages over other reporter gene systems in that chitobiase is not found in many cell lines traditionally used in reporter gene systems.

Finally, the forgoing examples are not intended to limit the scope of the present invention, which is set forth in the following claims. In particular, various equivalents and substitutions will be recognized by those of ordinary skill in the art in view of the foregoing disclosure, and these are contemplated to be within the scope of the present invention. All references cited herein are incorporated herein by reference in their entireties.